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On the detection of environmental effects on complex matrices combining off-line liquid chromatography and ^{1}H-NMR

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Abstract

An off-line combination of 400 MHz proton (^{1}H)-NMR spectroscopy and liquid chromatography (LC) has been used for the multi-component comparison of low-molecular weight compounds (i.e., chemical fingerprinting) in model fluid broths and (processed) tomato. The focus of the research described is on (i) devising GLP-like methods for sample handling and NMR measurements that will ensure reproducibility, (ii) an automated handling of data, (iii) validity of the designed methodology and (iv) the interpretation of large amounts of data.

Abbreviations: FID = free induction decay, GLP = good laboratory practise, LC = liquid chromatography, NMR = nuclear magnetic resonance, TMA = tetramethylammonium nitrate, TSP = 3-(trimethylsilyl)propionic-*2,2,3,3*-d_4 acid, sodium salt

Introduction

High resolution ^{1}H-NMR is well known for its ability to discriminate between numerous compounds in complex solutions (Ratcliffe 1991; Wasser et al. 1996; Van Den Thillart & Van Waarde 1996; Belton 1996; Bank 1997; Holmes et al. 1997; Vogels et al. 1993; Vogels et al. 1996a; Vogels et al. 1996b; Spraul et al. 1997; Noteborn et al. 1998). It is this feature of NMR which can be used to detect changes in complex matrices due to environmental effects. The positive aspects of ^{1}H-NMR as a fingerprinting method for chemicals are in fact: (i) a very broad detection method for nearly all low molecular compounds; (ii) a large dynamic range allowing a factor of 10,000 in difference in concentrations to be detected in one spectrum; (iii) a high degree of resolution enabling the detection of many compounds simultaneously; (iv) a means of identifying compounds by delivering structural information and (v) a tool for use in off-line and in-line combinations with separation techniques (Korhammer & Bernreuther 1996; Lenz et al. 1996; Scarfe et al. 1997; Sidelmann et al. 1997). However, apparent technological disadvantages are: (i) relative insensitivity requiring the need for concentrated samples; (ii) quality of spectra is dependent on operator's skill; (iii) pH dependence of signals when pH is near a pKa value; (iv) small salt dependency; (v) temperature dependency; and, (vi) too much information to handle if one is interested in all the chemical information available.

In order to get a maximum benefit out of ^{1}H-NMR as a technique for chemical fingerprinting it was a prerequisite to control the negative aspects and to improve conditions in order to enhance the positive aspects mentioned. Therefore GLP-like methods for the standardisation of sample treatment, NMR set-up and acquisition were considered as absolutely essential. The evaluation of large numbers of NMR spectra containing enormous amounts of information can only be dealt with by automation (Vogels et al. 1993; Vogels et al. 1996a; Vogels et al. 1996b; Spraul et al. 1997).

The strategy, here, to detect effects on changes in chemical compositions was directed initially towards locating statistically significant changes in spectra arriving from 2 populations, i.e., only a control and a

manipulated one. If significant spectral changes occurred, the next step was normally the identification and quantification of the compounds involved.

The research described consisted of four main parts: (i) to devise GLP like methods for sample handling and NMR measurements that will ensure reproducibility; (ii) to automate data handling; (iii) to validate the methodology designed and, (iv) to interpret large amounts of data.

Materials and Methods

Biological test samples

Cell culture media. Hepatocyte cell culture medium used as test samples for software development were derived from Waymouth medium MB 752/1 (Waymouth 1959) as follows: solution A, 0.2820 g of dry hepatocyte medium was dissolved in 10 ml of a 200 mM potassium phosphate buffer containing 1.00 mm of TSP and solution B, 0.2814 g of dry hepatocyte medium was dissolved in 9.1 ml of a 200 mM potassium phosphate buffer containing 1.00 mM of TSP. To these solutions spikes (small amounts) of 0.05 ml (4.2 mg/ml) phenylalanine, 0.10 ml (6.0 mg/ml) betaine and 0.75 ml (24.8 mg/ml) lysine-HCl solutions were added. The pH-values of solutions A and B was respectively 6.66 and 6.65. From both solution A and B seven (n = 7) NMR samples of 0.600 (±0.010) ml were prepared.

Plant tissues. In case of polar/water soluble compounds of, for instance, tomato fruit or juices, routinely 50 g batches of wet weight of plant tissue (n = 8) were grinded with liquid nitrogen, freeze-dried (2.5 g/batch), suspended in water (obtained by a Super-Q Plus water purification system, Millipore) (10% w/v) and stirred for 30 min at ambient temperature (Noteborn et al. 1995; Noteborn et al. 1998). The residue collected after filtration through a Sigma screen 40 mesh sieve was clarified by centrifugation and re-extracted. The supernatants were pooled (V_{tot} = 100 ml). After adjustment of the pH to 6.00 (±0.02) high molecular weight compounds (>10 kDa) were eliminated using ultrafiltration (Amicon Diaflow YM10 membrane). The apolar/membrane bound compounds were solubilised by taking routinely 20 g batches of wet weight of plant tissue (n = 8) after grinding with liquid nitrogen and extracting them with chloroform : methanol (2 : 1, v/v) for 20 min at ambient temperature (Noteborn et al. 1995; Noteborn et al. 1998). The chloroform layer was separated, clarified by centrifugation and evaporated. The aqueous plant tissue extract was fractionated by solid phase extraction (SPE) on a Bakerbond SPE Octadecyl (C18) support. The C18-supports were equilibrated with 500 mM $K_2HPO_4/KH_2P\text{-}O_4$ buffer (pH 6.00) and the tissue extracts separated by elution with 15 ml water (fraction A; SPE passed through fraction), 10 ml 20% acetonitrile (fraction B; SPE 20% acetonitrile eluate), 10 ml 50% acetonitrile (fraction C; SPE 50% acetonitrile fraction) and 20 ml 80% methanol containing 100 mM acetic acid (fraction D; SPE 80% methanol-100 mM acetic acid eluate). Fractions A to D were concentrated by lyophilisation. The chloroform/methanol extract was not fractionated into various fractions (fraction E; chloroform/methanol extract).

NMR samples

Standard test samples consisted of 0.100 (±0.005) mm TSP and 0.100 (±0.005) mm TMA in 0.600 (±0.010) ml 99.95% D_2O. Plant-derived tissue samples were dissolved as follows: fraction A in 4.00 ml D_2O containing 400 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.50) and 1 mM EDTA, fractions B, C and D were dissolved in 1 ml D_2O : acetonitrile-d_3 (1.00 : 1.00, v/v) containing 50 mm K_2HPO_4/KH_2PO_4 buffer and fraction E in 1 ml chloroform-d_4. High buffer capacities were used to guarantee pH equivalence between samples. All solvents contained 1.00 (±0.01) mM TSP as internal standard (A-D) or 1.00 (±0.01) mM tetramethylsilane (TMS, E). From all samples 0.600 (0.010) ml aliquots were analysed.

NMR set-up procedures

A constant volume of 0.600 (±0.010) ml of sample in high quality NMR sample tubes (5 mM, 535-PP-7, Wilmad, Buena, USA) was used to ensure optimal field homogeneity and optimal reproducibility of the magnetic field. Care was taken in the alignment during the exact positioning (tolerance within 0.5 mM) of the NMR-tube in the sample spinner. Apart from the normal sample to sample check of spectra characteristics concerning the NMR performance a separate check on NMR performance was done on a weekly basis in which the line width, line shape, signal to noise ratio and temperature were considered using a freshly prepared standard test sample (see above). The full line width at half height of the TSP resonance was shimmed down to 0.6–0.7 Hz without spinning

of the sample. The line shape of the TSP resonance was kept symmetrical and Gaussian. This criterium is subjective, however, and left to the expert judgement of the operator. The signal to noise ratio was calculated using the amplitude of the peak of TSP after careful tuning and shimming of the probe and pulse calibration. The ratio was found to be reasonably constant (within 10% of the average). Variations in signal to noise ratio were primarily attributed to line width differences. The temperature was kept constant for all samples (300.0 ($\pm$ 0.05) K). Temperature calibration was based on the difference in chemical shift between the HDO resonance and the TMA resonance. The variable temperature unit (temperature control) was tuned prior to measurement of the standard sample. The air flow was adjusted to 200 L/hr. The room temperature in which the NMR hardware was situated was regulated to 293 ($\pm$ 0.5) K. To confirm the temperature stability the deviation of the sample temperature as a function of time was measured periodically (interval of 1 to 15 min) acquiring a spectrum (1 scan) of the standard sample.

NMR data acquisition

^{1}H-NMR spectra were recorded at 400.13 MHz at 300.0 ($\pm$ 0.05) K on a Bruker AMX 400 widebore using a 5 mm probe. The spectra were acquired (reals and imaginaries simultaneous) in a "pseudo-oversampling" mode (G. Wider 1990). Data were processed after acquisition by special home written software. The spectrometer settings were: 0.2 s relaxation delay; ca 11 μs 60 degree pulse on ^{1}H; 12 5000 Hz spectral width; 200 free induction decays per experiment, averaged in 512 K memory; pre-scan delay = 100 $-$ 0.5 $\times$ (pulse length-dead time of pulse) μs. After acquisition the data were transformed to a smaller data set using blockwise summation of reals and imaginaries. This procedure led to the new data parameters respectively, data size = 16 K, spectral width = 5000 Hz and dwell time 100 μs.

Dataprocessing and software development

A Sun Sparc 2 Workstation was used. Programming was conducted in C, while the system was executed under Unix. To test the (sub)programmes various FID's were available and inspection of resulting spectra was performed by using the software package NMR1 (New Methods Research, Inc, East Syracuse, New York). The developed software package – i.e., automation scheme – for comparing 1D NMR spectra operated along a route according to following broad outlines. Preprocessing: step 1, zero filling (128 K, 0.038 Hz/point); step 2, Fourier transformation; step 3, phase correction (i.e., user predefined phase constants); step 4, automatic additional zero order phase correction (i.e., using marker TSP); step 5, positioning using marker and step 6, line width determination of marker TSP. Processing: step 1, correction of line width to 0.6 Hz using tuned exponential multiplication; step 2, standard/3 shifted sine bell filtering; step 3, zero filling (128 K, line width marker = 0.7 Hz); step 4, Fourier transformation; step 5, phase correction with user predefined phase constants; step 6, automatic additional zero order phase correction (i.e., using marker TSP); step 7, positioning and scaling using marker. Sets of peak positions and amplitudes were determined using following routines: step 1, iterative determination of peak and baseline zones; step 2, a series of baseline corrections using information from step 1; step 3, simultaneous peak search in 0.7 Hz regions in multiple spectra (i.e., first attempt in obtaining complete sets of peaks); step 4, elimination of noise artefacts in incomplete sets; step 5a, completion of sets by searching for shoulders; step 5b, completion of sets by backsearching in original data at known positions; step 6, completion of sets by searching and correction for pH dependent peaks (i.e., 10 Hz region) and step 7, check on individual peak variations in each population.

A module for the statistical interpretation of the previously described data reduction results was developed in such a way that peak-peak differences could be tested on various statistical significance levels (i.e., user defined, for example 99%, 95% etc.). In brief, the search for significant differences between two populations was performed in two ways: (i) using the amplitude data directly for statistics and (ii) using differences between local maxima and local minima for statistics. The latter required additional processing, i.e., a backsearch in the original data. This programme module also allows for a threshold for a user defined minimum difference i.e., relative to peak amplitude.

The procedures of NMR acquisition and data processing were validated (see below) using samples as described above.

Results and discussion

Preprocessing of data

Normally the quantification of NMR data will be done by integrating the area of a resonance peak. Related to this, amplitudes of peaks may be used as a measure for relative quantity if the line shapes of identical peaks in different spectra are the same. The differences observed in line shapes were mainly caused by differences in line width. Therefore, correction of the line width to a standard value of 0.6 Hz was applied using a tuned exponential multiplication at the cost of a small signal to noise variation. The information concerning the line width was calculated during the preprocessing.

In Figure 1 – amongst the effects of scaling and positioning of the marker – the effect of a tuned exponential multiplication is illustrated. As shown the data obtained from different spectra (n = 7) were made to fit each other nearly perfect.

Processing of data

After the tuned exponential multiplication the data were processed as described (see Materials and Methods). Because oversampling was used, the first order phase constant was practically zero. Standardised consecutively acquired spectra had small variations in the zero order phase constant. These variations were corrected by a separate programme, which calculated an additional zero order phase constant and performed the necessary corrections. The last step in this part of the processing of data was the shifting (i.e., positioning) and scaling of the spectra. This was necessary because of the dependency of proton frequencies on the deuterium lock frequency of D_2O (used in practically all samples), which in turn is in principle dependent on pH, salt concentration and temperature. Very slight differences in temperature and pH led to small shifts of entire spectra with regard to each other (see Figure 1).

Determination of sets of peak positions and amplitudes

Before amplitudes of peaks can be extracted a baseline correction (automated) is required (see Material and Methods). The determination of the baseline and peak zones was based on an iterative and heuristic approach starting with a definition of what should be expected for noise. The baseline correction itself was a series of linear corrections on regions determined as peak zones. After the baseline correction (see Figure 2) a multi-step simultaneous peak search in multiple spectra started, which correlated peaks to each other (see Materials and Methods).

Validation of data sets

The validity of the data sets containing peak information obtained through automation was checked by calculating the normalised confidence interval (significance level of 95%) of the normalised average. Figure 3 shows this normalised interval as well as the average amplitude as a function of peak number of the peak sets obtained from multiple spectra.

Peak sets were obtained from spectra of the hepatocyte cell culture media A and B. Approximately 500 peaks were found. Figure 3 shows that most peak sets had a 95% confidence interval within 10% of the peak average. Careful inspection of the spectra revealed that nearly all peak sets with larger confidence intervals descended from either small peaks or relatively small peaks near large peaks (partial overlap). Artefact peaks could easily be distinguished by their very large confidence intervals resulting from random variation in position and amplitude.

Statistical analysis of differences

The upper trace of Figure 4 shows the difference spectrum of medium B and A (randomly chosen spectra) in which prior to subtraction the positioning and the scaling of the internal reference was done, but not the tuned exponential multiplication. Considering the precautions which were taken prior to spectrum acquisition and the care taken in preparing the samples, there were still many extra amplitude differences. These extra differences had nothing to do with the spike added as shown in the middle trace of Figure 4. The magnitude of these differences were usually only a small percentage of the peaks they were derived from. They might be caused by a number of reasons i.e., slight differences in line shape (field homogeneity), small differences in pH or temperature, slight differences in pulse calibration, etc. Using the approach and software described above the lower trace was obtained. It is clearly shown that a nearly perfect representation of the spike was obtained. Even parts of the multiplet structure of the C_α and C_β proton resonances (respectively ca. 4.1 ppm and 3.2 ppm) of the minor spike of phenylalanine (20% of the original amount of phenylalanine) appeared; these resonances

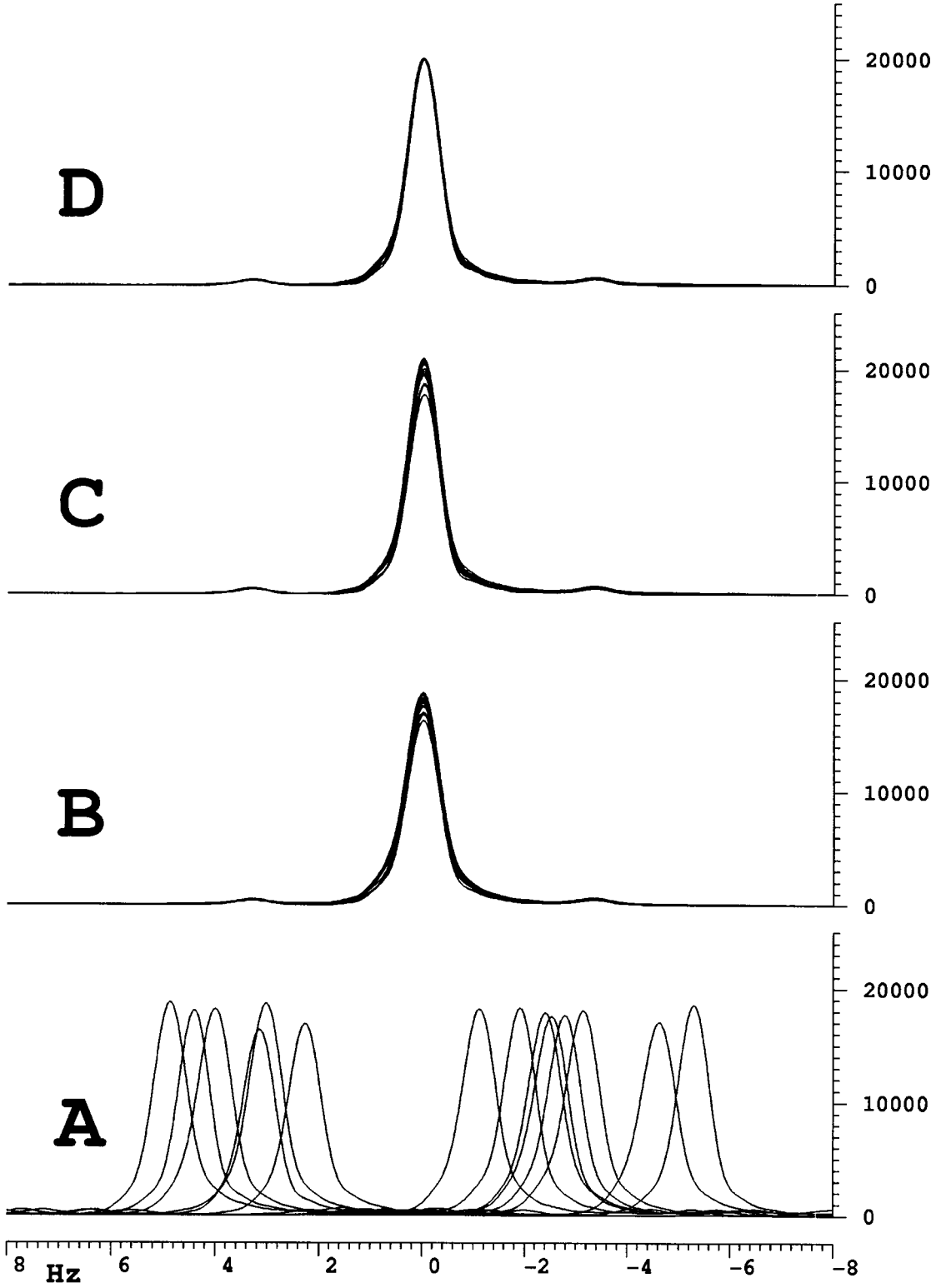

Figure 1. Overlays of regions of the baseline corrected ^{1}H-NMR spectra of the cell culture media A (n = 7) and B (n = 7) containing the resonance of the internal standard (TSP). A: normally processed uncalibrated data. B: as A after calibration (i.e., positioning/shifting of spectra). C: as B, but preprocessed using a tuned exponential multiplication. D: as C, all peaks scaled to that of the first spectrum (amplitude = 20.000).

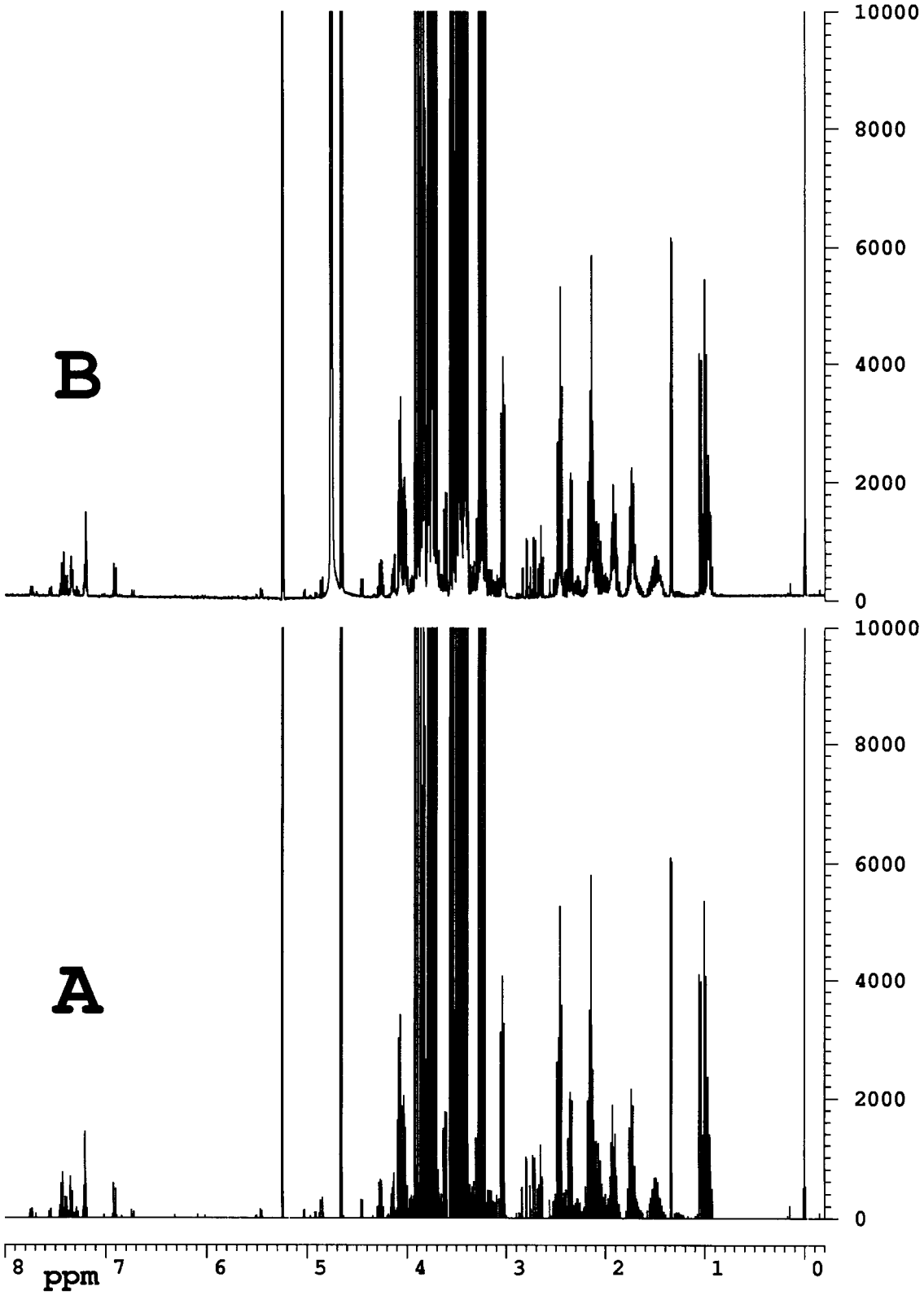

Figure 2. Example of a typical baseline correction and peak selection. A. Example of the baseline corrected ^{1}H-NMR spectrum of cell culture medium A. B. Result of peak selection of A.

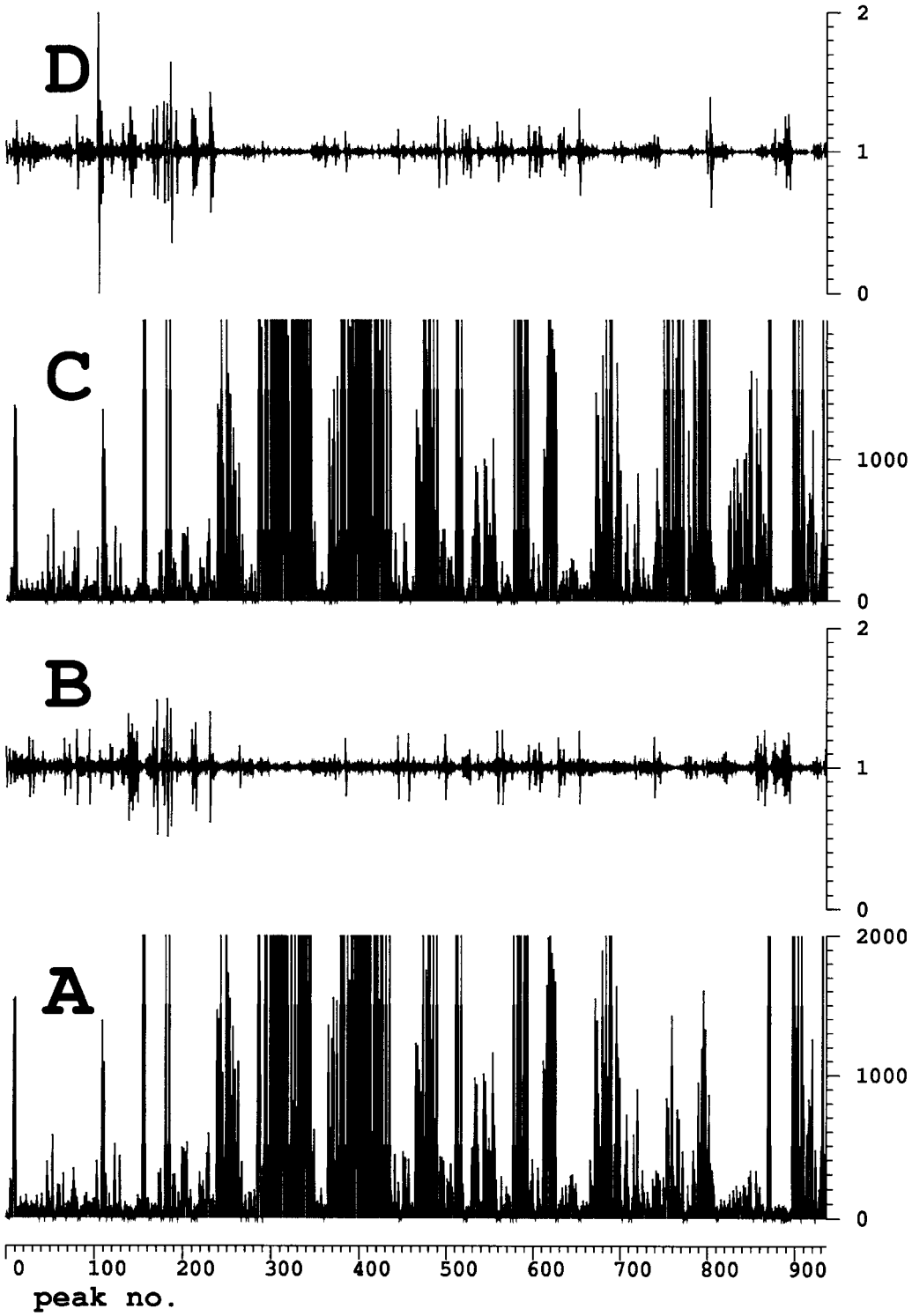

Figure 3. Examples of peak no. vs average amplitude and peak number vs. normalised confidence interval (95%). A. peak no. vs. peak average (n = 7) amplitude of cell culture media A; B. peak no. vs. normalised confidence interval (95%) of cell culture media A; C. as A for cell media B (n = 7); D. as B for cell media B (n = 7).

are in the most crowded part of the spectrum. Careful inspection of the spectra revealed that some very small real differences did not show up. The reason for this was a perfect overlap with large peaks impeding their traceability. Figure 4 provided an additional validation of the total approach. This result encouraged the testing of other more difficult biological samples as described below.

Testing biological samples

Biological samples such as plant tissues often contain compounds which may give rise to broader peaks. Furthermore, the numbers of compounds in these biological samples often exceed by far the number in cell culture media. Normally this may lead to problems in defining baselines. Tomato batches subjected to various environmental factors were used as testing material for the performance of the software on more complex matrices. Examples of the different samples (fractions) derived from the tomato batch are illustrated in Figure 5 (see Materials and Methods).

Although an extensive assignment of peaks to compounds was outside the scope of this study a very global compositional description follows here: fraction A, polar compounds, such as glucose and other monomeric and oligomeric sugars, amino acids and TCA-cycle substrates; fraction B, large variety of aromatic, aliphatic and sugar-like compounds; fraction C, primarily non-tomatine glycoalkaloids and aromatic compounds; fraction D, tomatines and aromatic compounds (p.e. indole-type compound) and fraction E, primarily lipids and carotenoids. Typically the detection limits in the various samples were around a few mg's per kg of wet weight of tomato.

The performance of the software is reflected in Figures 6 and 7. Figure 6 shows a total of ca. 2300 peaks in 5 spectra, representing probably a couple of hundred compounds. As can be deduced from Figures 5 and 6 the software can cope very adequately with full spectra, broader resonances and a more difficult baseline definition. Figure 7 shows the normalised confidence intervals (at a significance of 95%) of the normalised average of peak sets obtained from populations (n = 7) of the 5 fractionated tomato samples A through E. Obviously extensive sample handling (i.e., extraction, chromatography, etc.) induces deviations in concentrations of compounds and thus larger intervals than in the case of the neat cell culture media. Therefore, the results obtained here could be expected to be less well defined than in the case of the cell culture media A and B. It is clear from Figure 7, that there are relatively more artefacts or ill defined peaks in the tomato analysis than the cell media analysis; this is of course due to extensive sample handling. From the tomato analysis here and other similar tomato data (data not shown) it can be concluded, that differences exceeding at least 20% can normally still be detected.

Test cases

The first case was the influence of temperature (100 vs 70 °C) on the composition of tomato juice. Fraction A (100 °C) showed slightly higher glucose, much higher oligosaccharides and at least one other monosaccharide. Fraction A (70 °C) had slightly higher tyrosine, much higher valine, leucine and isoleucine and a much higher content of a sugar-like compound. These findings with regard to the amino acids are compatible with protease activity surviving the 70 °C treatment. In most cases of protease activity aliphatic and aromatic amino acids are cleaved preferentially. The differences in sugar profiles may be the result of enzymatic or temperature linked reactions. Since aliphatic amino acids and sugar-like compounds influence taste these findings are important. Fractions B showed clear differences in aromatic and sugar-like compounds. Fraction C (70 °C) contained slightly higher amounts of non-tomatine glycoalkaloids. Fraction D (100 °C) contained much higher levels (> factor 2) of tomatine glycoalkaloids and a slightly higher amount of an indole-type compound.

The second case studied the influence of external conditions with regard to the chemical composition of the tomato. Tomato samples obtained from the same seed divided into two tomato batches, but harvested in different seasons at approximately the same ripening stage, showed major numbers of differences, i.e., approximately 700 out of 2300 peaks (total of 5 fractions) were statistically different (99% significance). The differences were mostly above 50% and could range towards a factor 10 for some compounds. This indicated that about 30% of all compounds present differed in concentration. This clearly demonstrated that external factors such as culture, season, climate etc. have major effects on the overall chemical composition of a tomato.

Conclusions

Chemical fingerprinting using off-line LC-NMR is a powerful tool in obtaining information on possible

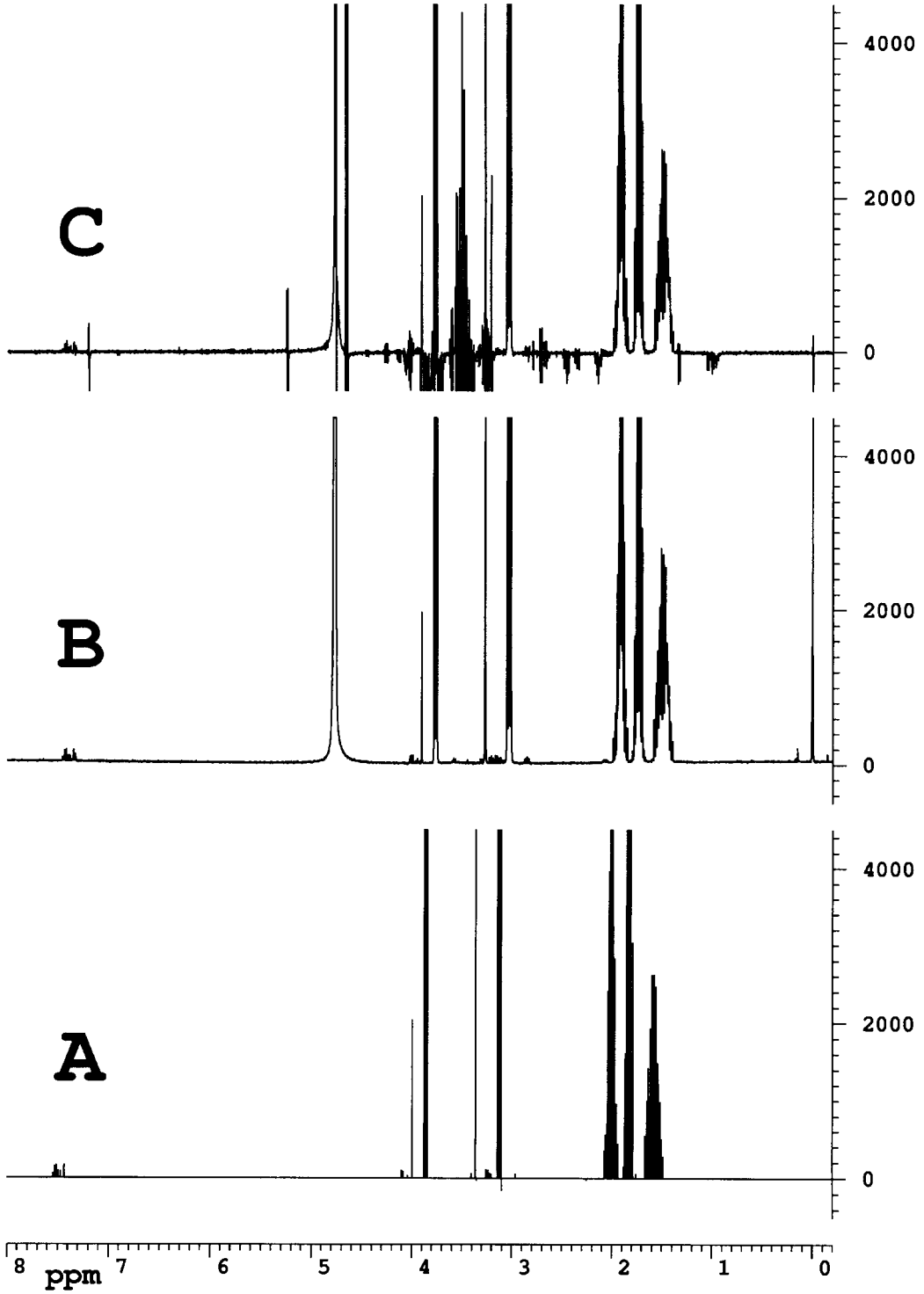

Figure 4. Spike detection in cell media B. A. difference ^{1}H-NMR spectrum of populations of spectra of cell medium A and B obtained as described in Materials and Methods using statistical analysis after data reduction (99% confidence; n = 7); a threshold was used of a minimum difference of 10% of the average amplitude; B. original ^{1}H-NMR spectrum of the spike and TSP; C. difference ^{1}H-NMR spectrum of randomly chosen spectra (positioned and scaled as described in the text) of cell medium A and B.

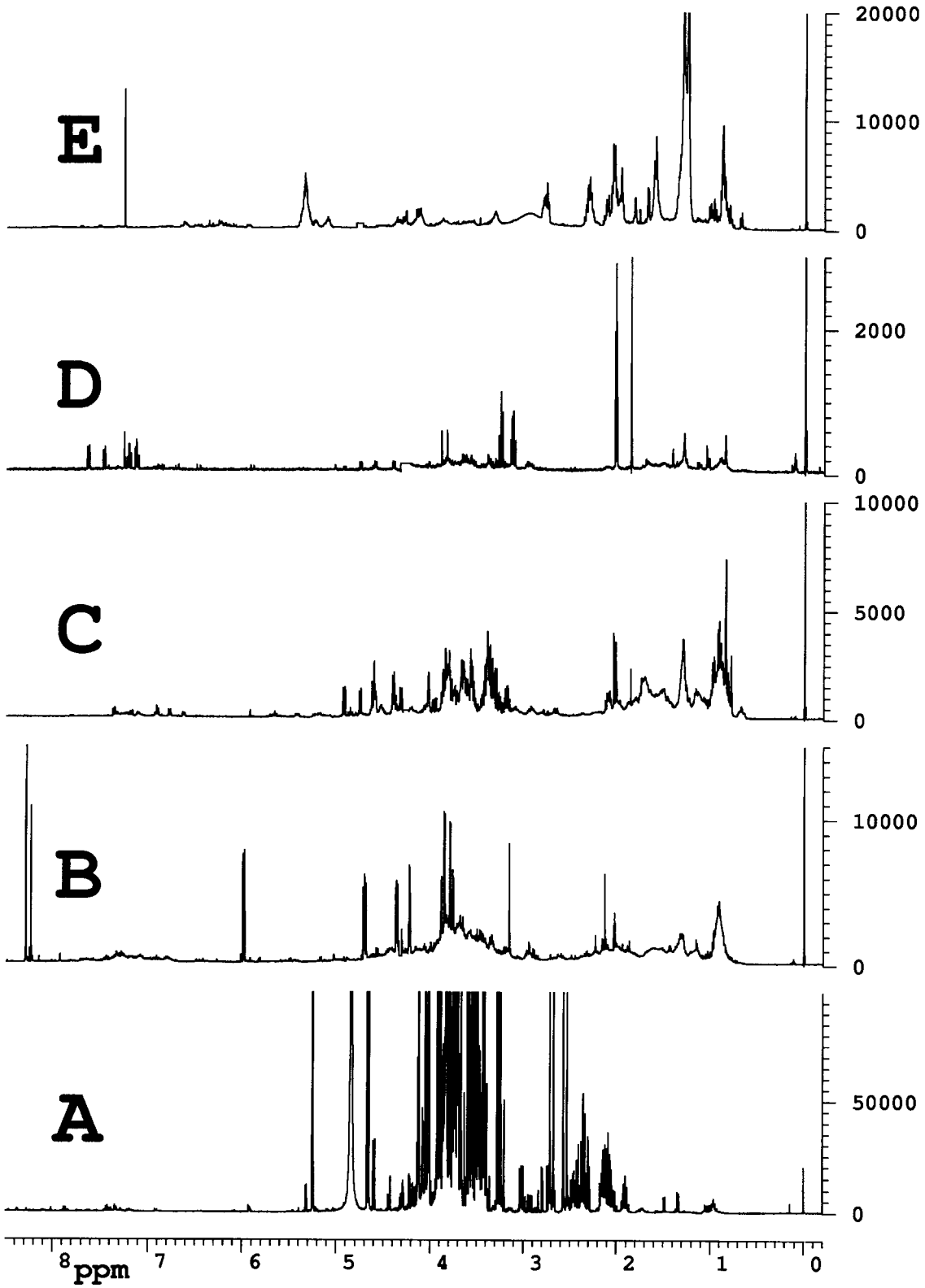

Figure 5. Examples of ^{1}H-NMR spectra of tomato fractions. Traces A through E correspond to, resp., the fractions A to E described in Materials and Methods.

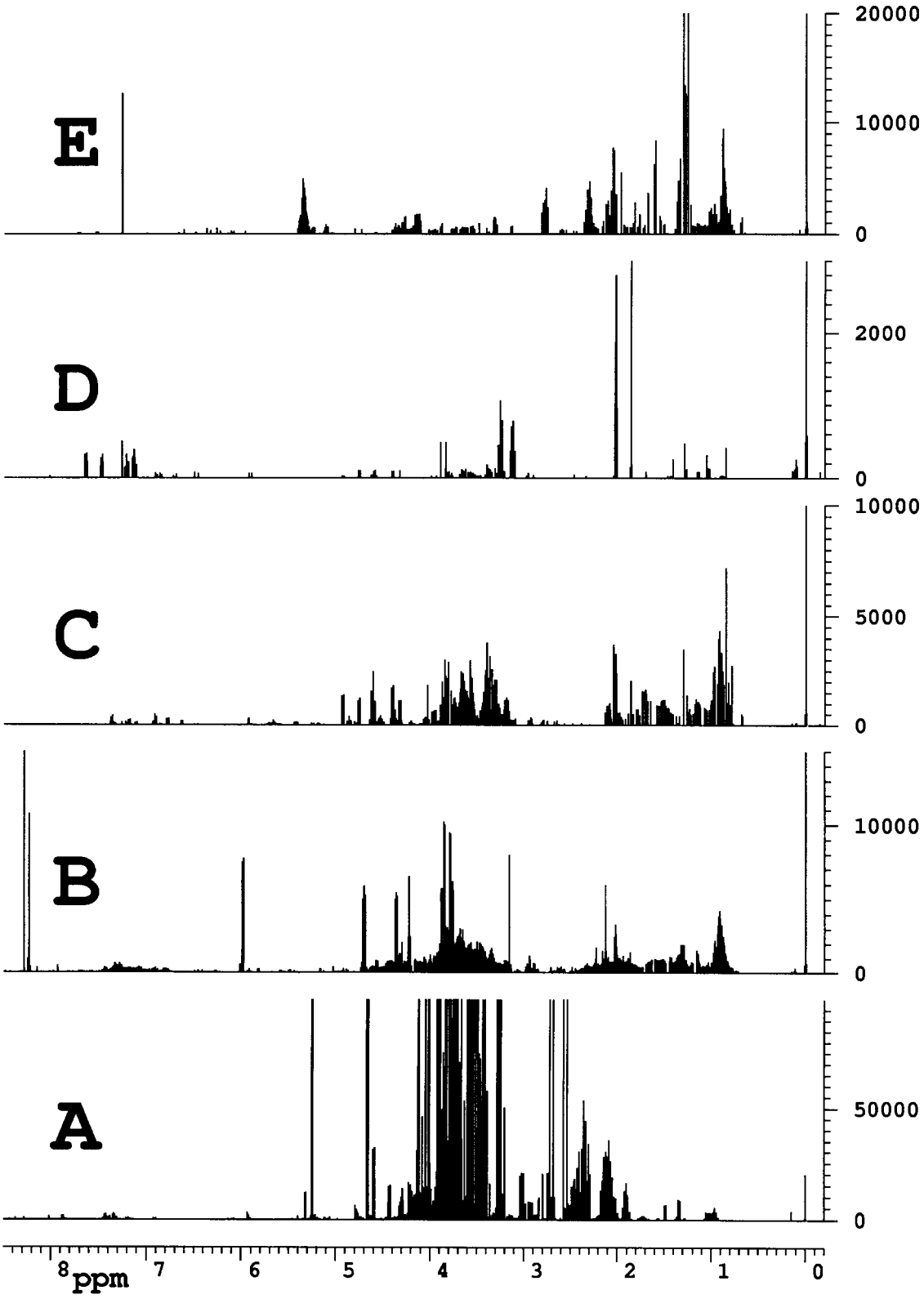

Figure 6. Examples of typical baseline correction and peak selection results obtained from the ^{1}H-NMR data (Figure 5) of tomato fractions. Traces A through E correspond to, respectively, the fractions A to E described in Materials and Methods.

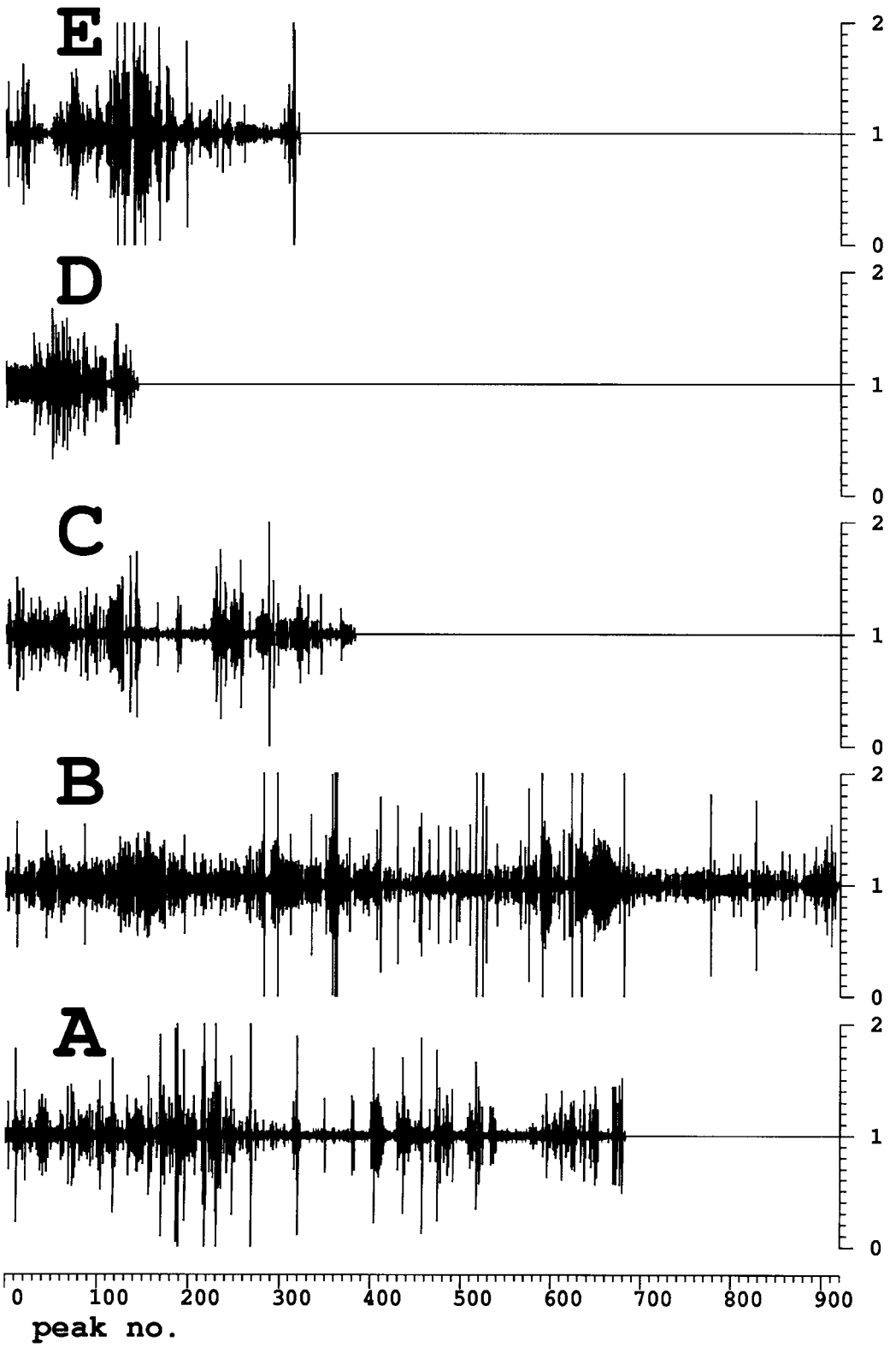

Figure 7. Peak no. vs normalised confidence interval (95%; n = 8) of the data shown in Figure 5 and Figure 6. Traces A through E correspond to, respectively, the fractions A to E described in Materials and Methods.

changes in composition of organisms or complex matrices in general. Assignment of the observed spectral changes to certain classes of compounds may indicate whether changes of (for instance toxicological) importance have taken place as a result of environmental influences. It is therefore helpful in the evaluation of effects of environmental factors such as downstream processing, climate, fertiliser etc. The methods described here are robust as long as GLP-like conditions for sample handling, data acquisition and automation of data handling are maintained. In the future when more than 2 populations of sets of spectra have to be compared, this type of research will benefit from multivariate analysis techniques.

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